

Gene Structure and Expression of a Pyrethroid-Metabolizing Esterase, CzEst9, from a Pyrethroid Resistant Mexican Population of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae)

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ABSTRACT A population of *Rhipicephalus (Boophilus) microplus* (Canestrini) (Acari: Ixodidae), designated Coatzacoalcos, sampled from a ranch near Veracruz, Mexico, was found to possess a high level of resistance to pyrethroid-based acaricides. Bioassay and biochemical and molecular analysis had previously shown that resistance in this population could primarily be attributed to expression of a highly active metabolic esterase designated CzEST9. We cloned and sequenced the entire CzEST9 coding region, including introns and >1.0 kb upstream from the transcription start site, and we compared the upstream region sequence between individual resistant and susceptible ticks from several populations with different pyrethroid resistance characteristics. In the 1.0-kb upstream region sequence, four variant nucleotides were found, and a TGA trinucleotide occurred as either four, five, or nine tandem repeats. However, none of these promoter region sequence differences could be clearly associated with a pyrethroid-resistant phenotype; thus, we concluded that differences in gene promoter sequence were not responsible for the pyrethroid resistance mechanism in the Cz strain. CzEST9 was expressed in recombinant *Escherichia coli* and *Pichia pastoris* systems and esterase activity was obtained in recombinant CzEST9 from the *P. pastoris* system.

KEY WORDS *Boophilus microplus*, pyrethroid resistance, metabolic esterase, recombinant protein expression

Boophilus ticks present serious problems to cattle producers in many parts of the world, due both to direct effects of tick infestation and indirect effects from the pathogens these ticks transmit to infested cattle. The negative impacts to the U.S. cattle industry were such that an intensive 55-yr U.S. Department of Agriculture (USDA) program was conducted to eradicate *Boophilus* ticks from the country, and the United States remains *Boophilus*-free through a USDA-APHIS/VS quarantine program established at the Texas border between the United States and Mexico, where these ticks are endemic and problematic (Graham and Hourigan 1977). The major control methodology for *Boophilus* in Mexico centers around chemical acaricides, and the pyrethroid class of acaricides has played a major role in these control efforts. Pyrethroid-resistant populations have been reported in Mexico (Fragoso et al. 1995; Rosario-Cruz et al. 2005), and studies of resistance mechanisms have reported target site

resistance and metabolic enzyme activities as major causes of pyrethroid resistance in Mexican *Boophilus* ticks (Miller et al. 1999; Jamroz et al. 2000).

Jamroz et al. (2000) reported the interesting finding of a highly active putative carboxylesterase, designated EST9, in the Coatzacoalcos (Cz) strain of *R. microplus*. This strain seemed to have a significant amount of metabolic esterase-based resistance to pyrethroids and further work led to the purification of CzEST9 (Pruett et al. 2002) and the characterization of the *CzEst9* transcript expression pattern in several strains with differing levels of pyrethroid resistance (Hernandez et al. 2002). Hernandez et al. (2000) had reported previously the Cz strain contained elevated copy numbers of a gene corresponding to a cDNA designated as clone 13, which Pruett et al. (2002) later confirmed as encoding CzEST9. Two alleles of *CzEst9* were reported whose translation products differed at a single amino acid position (Asp374 → Asn), and the Asn-containing allele was present in 100% of the Cz individuals and to a much lower degree in other *R. microplus* strains (Hernandez et al. 2000). Guerrero et al. (2002) reported the Asn-containing allele made a statistically significant contribution toward permethrin resistance compared with the Asp-containing allele, but the likely major resistance mechanism of the Cz strain was due to the overall higher amount of CzEST9 enzymatic activity in Cz compared with other

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Table 1. *R. microplus* strain information

Strain	Geographical origin	Resistance phenotype ^a		Generation used	Reference ^b	
		Permethrin	Coumaphos		Permethrin	Coumaphos
Cz	Coatzacoalcos, Veracruz, Mexico	R	R	f14, f36	Guerrero et al. 2001	Li et al. 2003
Corrales	Colima, Colima, Mexico	R	S	f14	Guerrero et al. 2001	Li et al. 2003
Deutch	Webb County, TX	S	S	f8	Unpublished	Unpublished
Fernandez	Hidalgo County, TX	S	S	f1	Guerrero et al. 2001	Unpublished
Munoz	Zapata County, TX	S	S	f10	Unpublished	Li et al. 2003
La Minita	Starr County, TX	S	S	f9	Guerrero et al. 2001	Unpublished
Pena	Starr County, TX	S	S	f1	Guerrero et al. 2001	Unpublished
Rio Bravo	Rio Bravo, Tamaulipas, Mexico	R	S	f1	Unpublished	Unpublished
San Felipe	Soto la Marina, Tamaulipas, Mexico	R	S	f28	Guerrero et al. 2001	Li et al. 2003
San Roman	Champonot, Campeche, Mexico	R	R	f2	Unpublished	Li et al. 2003
Tuxpan	Tuxpan, Vera Cruz, Mexico	S	R	f20	Guerrero et al. 2001	Li et al. 2003

^a Determined by larval packet test bioassay. S, susceptible; R, resistant.

^b Publication reporting larval packet test bioassay results for the strain; Unpublished indicates strain has been analyzed by larval packet test bioassay, but results have not been published.

strains. Recently, Baffi et al. (2007) reported that a mutant esterase with very high DNA sequence identity to *CzEst9* has been associated with acaricide resistance in Brazilian populations of *R. microplus*. To investigate the role of transcriptional regulation in the mechanism of pyrethroid resistance in this interesting esterase of *R. microplus*, we have cloned and sequenced the entire coding region of the gene, including the introns and 5'-untranslated region. We have compared the 5' upstream region from individual *R. microplus* ticks from strains with differing levels of pyrethroid resistance. Although some nucleotide differences were noted between individual ticks, these differences could not be correlated with pyrethroid resistance. Finally, we have used a *Pichia pastoris* recombinant protein expression system to express active CzEST9 and biochemically verified the esterase activity of the expressed recombinant protein.

Materials and Methods

Tick Rearing. Ticks were reared at the USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX, as described by Davey et al. (1980). Cz

has high metabolic esterase-based resistance to pyrethroids (Miller et al. 1999, Jamroz et al. 2000) and low metabolic resistance to organophosphates (Li et al. 2003), and it was collected in 1994 near Coatzacoalcos, Mexico. Data for all strains used, including origin and resistance to coumaphos and permethrin, are noted in Table 1. Although inbred, these tick strains are not genetically homogeneous, and tick larvae from the Cz strain were treated with 3.75 or 1.88% permethrin in larval filter packet bioassays (Guerrero et al. 2002) to segregate permethrin-resistant Cz individuals from susceptible individuals. Larvae from the SF strain were treated with 3.75% permethrin and segregated similarly.

DNA and RNA Manipulations. Primer sequences from various protocols are listed in Table 2. To clone the *CzEst9* upstream promoter region, genomic DNA was isolated from eggs from the f36 and f10 generations of the Cz and Munoz strains, respectively, following the protocol in Sambrook et al. (1989) for isolation of very high molecular weight DNA. Genomic libraries were synthesized using the Universal GenomeWalker kit (Clontech, Mountain View, CA) and screened per the manufacturer's protocol by

Table 2. Sequences of various primers

Primer	Sequence	Description	Annealing site ^a
FG274 ^b	5'-TAGTACCACTGCCGAAACCAGTCAGTAG-3'	Genomic DNA clone 1° PCR primer	5710-5725, 15530-15541
FG275	5'-AATAGCGATGCGCTGGCTGCGCTGCTCACA-3'	Genomic DNA clone 1° PCR primer	5634-5663
FG290	5'-GCCAGGATAACTGCTGG-3'	Transcription start mapping Upstream primer for promoter amplification	224-240
FG291	5'-TTTGCGTTCGAGAAGAACC-3'	Downstream primer for promoter amplification	659-677
FG292	5'-TTAAGATCTATGCATCGCC-3'	Upstream primer for promoter amplification	793-811
FG302	5'-GGAAGTTAACCTCCTAGAC-3'	Downstream primer for promoter amplification	1037-1055
FG328	5'-CATGCGGTGAAAGCAGCTGT-3'	Upstream primer for coding region amplification	15547-15567
FG329 ^c	5'-AGGTTAGGTACCAAGACTGACTTCCAGCGCTC-3'	Downstream primer for coding region amplification	26509-26528

^a Numbering based on Deutch BAC clone GenBank accession no. DQ533868.

^b Interrupted by intron in genomic sequence

^c 5' end of primer is six random nucleotides and the KpnI recognition sequence for subcloning into *Pichia* expression vector.

using primer FG274 for the primary polymerase chain reaction (PCR), FG275 for the nested PCR, and the Advantage Genomic Polymerase Mix (Clontech). FG274 and FG275 anneal to the 5'-untranslated region of *CzEst9*, and they were paired with the adaptor-primers from the kit to synthesize DNA containing the upstream promoter region. The primary PCR was carried out using a DNA Engine (MJ Research, Watertown, MA) programmed for 95°C for 1 min followed by six cycles, each consisting of denaturation at 94°C for 2 s and extension at 72°C for 3 min, followed by 36 cycles, each consisting of denaturation at 94°C for 2 s and extension at 64°C for 3 min. A final extension step at 64°C for 4 min was included. The nested protocol consisted of 95°C for 1 min followed by four cycles, each consisting of denaturation at 94°C for 2 s and extension at 72°C for 3 min followed by 19 cycles, each consisting of denaturation at 94°C for 2 s and extension at 64°C for 3 min. The program also included a final extension step at 64°C for 4 min. Reaction products were fractionated on agarose TBE gels, and DNA was visualized by staining with GelStar DNA staining dye (FMC Bioproducts, Rockland, ME) followed by UV illumination. The predominant amplification product was excised from the agarose, purified by the QIAquick gel extraction kit (QIAGEN, Valencia, CA), cloned into the *SrfI* site of pPCR-Script Amp SK+ (Stratagene, La Jolla, CA), and sequenced with Big-Dye terminator reactions and the Prism 3100 genetic analyzer according to manufacturer's instructions (Applied Biosystems, Foster City, CA).

Double-selected polyA RNA was obtained from fl4 *Cz* larvae by using the PolyAPure kit (Ambion, Austin, TX) and primer extension analysis used to identify the *CzEst9* mRNA start site. SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) was used with ³²P end-labeled primer FG275 and 500 ng of polyA RNA according to the SuperScript protocol. Extension products were analyzed on standard 5% DNA sequencing gels by using sequencing reaction products from a *CzEst9* genomic clone from the *Cz* strain containing the transcript coding and upstream promoter regions as a size ladder marker to identify the nucleotide corresponding to the transcript start site.

Upstream promoter region sequences were examined from individual larvae from various *R. microplus* strains. Briefly, genomic DNA was purified from individual larvae by an adaptation of a method used for DNA purification from *Drosophila melanogaster* (Meigen) (Czank 1996). A disposable pellet pestle for 1.5-ml centrifuge tubes (Kontes Glass, Vineland, NJ) was used to crush and grind individual larvae. Twenty-five microliters of sample buffer (100 mM Tris, pH 8.3, and 500 mM KCl) was added to the tube, and grinding continued for ≈20 s. The tube contents were briefly microcentrifuged and placed in a boiling water bath for 3 min. After cooling, 1 μl of the DNA solution was used for PCR with a reaction mixture containing 1 μM each primer, 10 mM Tris(hydroxymethyl)aminomethane hydrochloride, pH 8.3, 50 mM KCl, 0.05 mM each dNTP, 2 mM MgCl₂, and 0.1 μl of a 1:1 (vol:vol) mix of *AmpliTaq* DNA Polymerase (5 U/μl stock; Ap-

plied Biosystems) and TaqStart antibody (1.1 μg/μl stock; Clontech). The thermocycling program used an initial denaturation step of 96°C for 2 min followed by 35 cycles, each consisting of denaturation at 94°C for 1 min, annealing at either 65°C (primer FG290-FG291 combination) or 61°C (primer FG292-FG302 combination) for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Amplification products were cloned and sequenced as described previously. MacVector 7.2 (Accelrys, San Diego, CA) was used to search for transcription factor motifs using the supplied transcription factor subsequence motif file (Ghosh 1990).

BAC clone Bm91N14 was isolated by hybridization of ³²P-labeled *CzEst9* cDNA to filter replicas of an *R. microplus* Deutch strain bacterial artificial chromosome (BAC) library (Amplicon Express, Pullman, WA) by using standard protocols (Sambrook et al. 1989). Sequencing and contig assembly was performed at TIGR and the sequence reported herein deposited in GenBank (accession no. DQ533868). BAC sequence was used to locate positions and sequences of introns within the *CzEst9* transcript.

Recombinant Protein Expression. The EasySelect *Pichia* Expression kit (Invitrogen) was used to express recombinant *CzEST9* in *P. pastoris*. The *CzEst9* coding region from bp 147 to 1778 (GenBank accession no. AF182283), which includes all amino acids except for the final Phe, was amplified from plasmid containing the complete cDNA by using PfuTurbo Hotstart DNA Polymerase and primers FG328 and FG329, gel-purified product digested with KpnI to remove the seven 5'-most nucleotides supplied by FG329. EcoRI linker adapters (Promega, Madison, WI) were ligated onto the EcoRI sticky end 5' to the N-terminal encoding side of the cDNA to enhance ligation to the vector. The pPICZαA vector was digested with EcoRI and KpnI, ligated to the *CzEst9* fragment, transformed into TOP10 competent cells (Invitrogen), and transformants containing inserts verified by sequencing to ensure both the 5' and 3' ends of the *CzEst9* coding regions were in-frame with the initiator Met of the vector's α-factor signal sequence and the C-terminal polyhistidine tag and stop codon. Recombinant DNA was transformed using supplied protocols for electroporation of freshly prepared competent *P. pastoris* from the GS115 and KM71H strains. A GS115 clone was selected for expression experiments conducted using buffered methanol-complex medium or minimal methanol histidine medium (BMMY or MMH from kit protocols), and both secreted and intracellular proteins were analyzed for the presence of *CzEST9*-associated esterase activity by using native esterase activity gel electrophoresis as described in Pruett et al. (2002). Western blotting was performed using the XCell SureLock Mini-Cell and Blot Module, Western-Breeze Chromogenic Immunodetection kit, and anti-His (C-term)-horseradish peroxidase (HRP) antibody (Invitrogen) to verify esterase activity was associated with a poly-His-tagged protein of molecular weight expected from the pPICZαA-*CzEst9* recombinant construct. A positive control containing

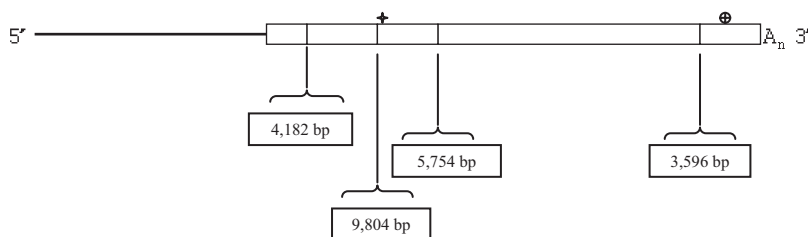


Fig. 1. Schematic of *CzEst9* structure. Exons and upstream promoter region are denoted approximately to scale. Exons 1–5 sizes are 222, 292, 288, 1,223, and 224 bp, respectively. Sizes of introns 1–4 are not to scale and contain 4,182, 9,804, 5,754, and 3,596 bp, respectively. Transcription start site is nt 1,030 and site of polyadenylation is nt 26,614. The locations of the ATG initiator methionine (+, nt 15,548–15,550) and TAA stop codons (+, nt 26,530–26,532) are indicated above their respective exons. This structure is based on BAC clone sequence from the Deutch strain, GenBank accession no. DQ533868.

active *CzEST9* for use during activity gel analyses was obtained from total protein extracts of Cz strain larvae as described in Jamroz et al. (2000). A negative control was provided by *P. pastoris* transformants containing pPICZαA without insert. A positive control for induction of recombinant expression was provided with the *P. pastoris* kit (strain GS115/pPICZ/lacZ Mut⁺ β-galactosidase), which expressed a 119-kDa intracellular fusion protein containing β-galactosidase fused at the C terminus to the *myc* epitope and a polyhistidine tag. The ProBond Purification System (Invitrogen) was used for purification of polyhistidine-containing recombinant protein under native conditions. All expression constructs were verified by sequencing both strands of the *CzEst9* insert and all vector–insert junctions.

Results and Discussion

Gene Structure. We sequenced a *CzEst9*-containing BAC clone from the Deutch pyrethroid-susceptible strain and genomic DNAs from the susceptible Munoz strain and the pyrethroid-resistant Cz strain, which contained the *CzEst9* upstream gene promoter and 5′-untranslated regions. Figure 1 depicts the *CzEst9* gene structure as found by sequencing of the Deutch strain BAC clone. GenBank accession no. DQ533868 contains 26,713 bp, including the entire transcript sequence and 1,029 bp of upstream promoter sequence. To determine the transcript start site, we used polyA RNA from the Cz strain in conjunction with primer extension analysis, and we found the transcript starts at nt 1,030 and the 5′-untranslated leader is 533 nt. The sizes of the five exons are 222, 292, 288, 1,223, and 224 bp, whereas the four intron sizes are 4,182, 9,804, 5,754, and 3,596 bp. Another interesting intron feature is that intron 2 contains three inverted copies of intron seven from the putative *R. microplus* glucose 6-phosphate dehydrogenase gene (DQ118973). Intron 3 contains two copies, one of which is inverted, of the same glucose 6-phosphate dehydrogenase intron. Positions 23,242–24,755 (intron 4) contain 528 CA repeats in this 1,514-nt region. The initiator ATG codon starts at position 15,548, which is located in the third exon, and the TAA stop codon starts at position 26,530. A polyadenylation signal motif is located at nt 26,595, and the

polyadenylation site is at nt 26,614. The 3′-untranslated region extends from nt 26,530 to nt 26,614. The first intron found in the Deutch strain BAC sequence is absent from the genomic fragments sequenced from both the Munoz and Cz strains. Introns 2–4 have the canonical GT . . . AG sequence at the 5′ and 3′ end splice sites. Intron 1 has a CC . . . AG; however, a GT is present at nt 13–14 of intron 1 and perhaps this splice site is used, which would create an additional 13 nucleotides in the 5′-untranslated region of the unprocessed *CzEst9* transcript from the Deutch strain compared with the Munoz and Cz strains. Differences in *CzEst9* gene copy number between *R. microplus* strains have been documented (Hernandez et al. 2000), and it is possible that differences at the gene structure level also exist. The BAC clone source is the Deutch strain, whereas the cDNA sequences, which are being used to identify introns 1 and 2, are derived from the Munoz and Cz strains. The Deutch strain exons and the published *CzEst9*-encoding clone 13 cDNA sequence from the Cz strain (Hernandez et al. 2000) are nearly identical, having only three synonymous nt differences in the coding region and two nt differences in the 5′-untranslated region. However, it is possible that the Deutch strain expresses functional *CzEST9* yet contains strain-distinctive sequences in other noncoding regions of *CzEst9*. There are no nucleotide differences between the *CzEST9* protein coding regions of this BAC clone-derived sequence from the Deutch strain of *R. microplus* and that from the original *CzEst9* cDNA clone derived from the Gonzalez strain of *R. microplus* (AF182283).

The sequence of 1.0 kb from the upstream gene promoter region and 0.45 kb from the 5′-untranslated region of *CzEst9* was determined from genomic DNA isolated from the Munoz and Cz strains (Fig. 2). The sequences from these regions were found to be nearly identical in the Munoz (GenBank accession no. DQ533869), Cz (GenBank accession no. DQ533870), and Deutch strains of *R. microplus*. A cap signal initiator sequence of CCTGTTT at nt 1,029–1,036 fits well with the (t/g)C(A/t)(g/t/c)(T/c/a)(c/t)(t/c/g)(t/c) –2 to +5 position consensus noted in the Eukaryotic Promoter Database Release 86 (Schmid et al. 2006; www.epd.isb-sib.ch). Several other sequence motifs were found in the upstream region, including

The only nucleotide differences in the upstream promoter regions were found at nt 301, 325, 939, and 969 (Table 3; nucleotide numbering corresponds to

Table 3. Sequence of variable region of *CzEst9* promoter from individual pyrethroid-resistant or -susceptible larvae

Larval ID	TGA repeat region ^a	nt 301 ^b	nt 325 ^b	nt939 ^b	nt 969 ^b
Munoz susceptible genomic library clone sequence	(TGA) ₉ TGGTGATACTGA	T	A	T	G
Cz pyrethroid resistant genomic library clone sequence	(TGA) ₅ TGGTGATACTGA	G	G	C	T
Deutch susceptible BAC library clone sequence	(TGA) ₉ TGGTGATACTGA	T	A	T	G
Pyrethroid-susceptible larvae					
Cz Dead #3	(TGA) ₅ TGGTGATACTGA	G	A	C	T
Tuxpan #29	(TGA) ₅ TGGTGATACTGA	G	A	C	T
Cz Dead #20	(TGA) ₅ TGGTGATACTGA	G	A	C	T
Cz Dead #26	(TGA) ₅ TGGTGATACTGA	G	A	C	T
Cz Dead #12	(TGA) ₅ TGGTGATACTGA	G	A	C	T
Cz Dead #14	(TGA) ₅ TGGTGATACTGA	G	A	C	T
Cz Dead #15	(TGA) ₅ TGGTGATACTGA	G	A	C	T
SF Dead #26	(TGA) ₅ TGGTGATACTGA		(Not determined)		
Fernandez #29	(TGA) ₉ TGGTGATACTGA		(Not determined)		
La Minita #29	(TGA) ₉ TGGTGATACTGA	T	A	T	G
Pena #29	(TGA) ₉ TGGTGATACTGA	G	A	T	G
SF Dead #15	(TGA) ₉ TGGTGATACTGA	T	A	T	G
SF Dead #28	(TGA) ₉ TGGTGATACTGA	T	A	T	G
Pyrethroid-resistant larvae					
Corrales #29	(TGA) ₅ TGGTGATACTGA	G	A	T	G
Cz Alive #13	(TGA) ₅ TGGTGATACTGA	G	A	T	G
Cz Alive #21	(TGA) ₅ TGGTGATACTGA	G	A	C	T
SF Alive #25	(TGA) ₅ TGGTGATACTGA	G	A	C	T
San Roman #29	(TGA) ₄ TGGTGATACTGA	G	A	C	T
Rio Bravo #29	(TGA) ₅ TGGTGATACTGA	G	A	T	G
	(TGA) ₉ TGGTGATACTGA				
SF Alive #9	(TGA) ₉ TGGTGATACTGA	T	A	T	G

^a bp 419–445 in Deutch BAC clone (GenBank accession no. DQ533868) and Munoz genomic library clone sequence (GenBank accession no. DQ533869), and bp 419–443 in Cz genomic library clone sequence (GenBank accession no. DQ533870).

^b Based on Deutch BAC library clone numbering (GenBank accession no. DQ533868) as varying TGA repeat number affects nucleotide numbering post-TGA region.

sequences of Deutch BAC clone DQ533868 and Munoz clone DQ533869). The TGA trinucleotide at nt 419 was repeated nine times in both the Deutch and Munoz clones (nt 419–445) and only five times in the Cz genomic clone (nt 419–433). The sequence at the four single nucleotide polymorphic sites and the TGA repeat region also was determined in single larvae, which had been phenotyped for pyrethroid resistance or susceptibility (Table 3) by exposure to pesticide or according to strain characteristics as determined in previous studies by bioassays (Table 1). No clear trends that could be associated with pyrethroid resistance were seen in these promoter region sequence differences. For example, two individuals were sequenced from the pyrethroid resistant Rio Bravo strain and both the (TGA)₅ and (TGA)₉ forms were found. Although a (TGA) transcription factor binding motif was not seen in the Eukaryotic Promoter Database, it is possible that four additional (TGA) repeats could cause the DNA helix of the upstream promoter region to adopt a conformation with a significantly different presentation of transcription binding factors, resulting in altered *CzEst9* transcription activity. We attempted to study this possibility further by segregating dead and alive larvae from the Cz and San Felipe resistant strains after exposure to a moderate dose of pyrethroid and sequencing the (TGA) repeat region. However, it is likely that the inbreeding from the laboratory rearing procedure has resulted in homozygosity at the (TGA) repeat site of the Cz strain,

because all sequenced Cz individuals possessed the (TGA)₅ genotype irrespective of whether they survived or were killed by the pyrethroid exposure. One nucleotide difference in the 5' untranslated region of the genomic DNAs from these three strains was that the C at nt 1399 of the Munoz genomic DNA is a T at the equivalent position in both the Deutch BAC (nt 5581) and Cz (nt 1387) genomic fragment sequence.

Thus, the evidence suggests that promoter differences are not responsible for the overexpression of *CzEST9* protein (Jamroz et al. 2000) and mRNA (Hernandez et al. 2002) in the pyrethroid-resistant strain. Hernandez et al. (2000) reported increased copy number of clone 13 esterase in the Cz strain compared with five other strains of *R. microplus*. Because Pruett et al. (2002) confirmed clone 13 encoded *CzEST9*, increased gene copy number may be responsible for the overexpression of *CzEST9* in the Cz strain. It is also possible the *CzEst9* promoter sequenced in this study may differ from the promoter of a different gene copy of *CzEst9* in the Cz strain that does possess an altered transcriptional control element, which results in overexpression.

Expression of Recombinant *CzEst9*. In preliminary experiments, we experienced difficulties in expressing enzymatically active *CzEST9* in *Escherichia coli*, despite considerable effort to express under different growth and inducing conditions and using expression constructs with different upstream coding sequences in the vector in an attempt to transport the expressed

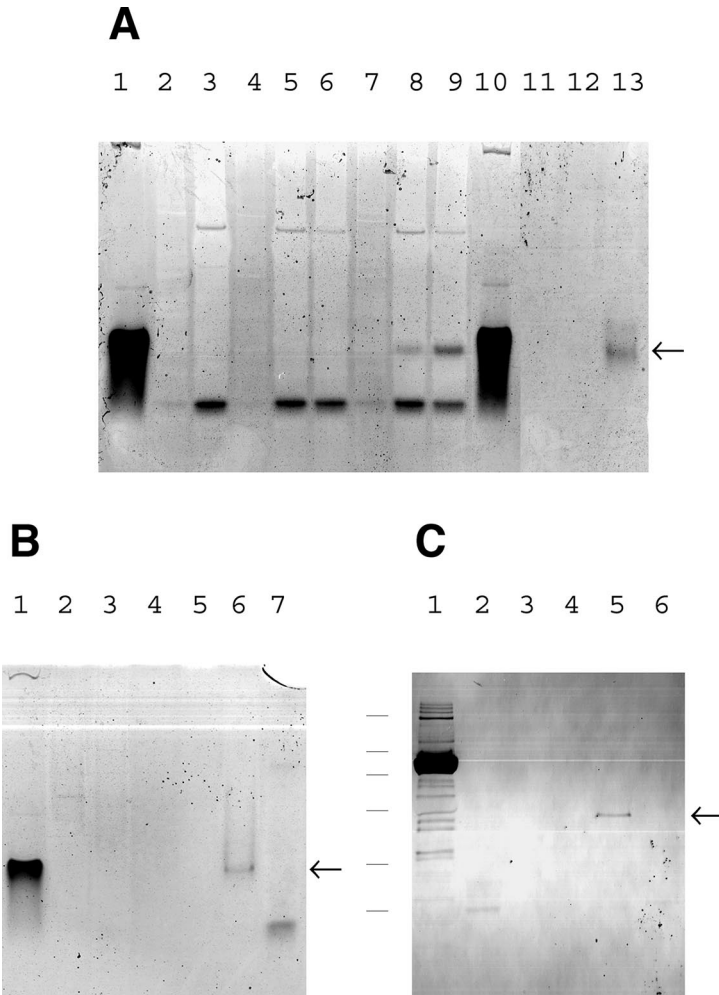


Fig. 3. Expression of recombinant CzEST9 in various expression conditions. Arrows note the location of the expected recombinant product. (A) Native esterase activity gel of *CzEst9*-pPICZ α A expression in *P. pastoris* GS115 after growth in MMH (lanes 2–9) or BMMY (lanes 11–13) media. Total protein from equal amounts of yeast cell equivalents (1.5×10^6 cells) were loaded per lane. Lanes 1 and 10, total soluble native proteins from Cz strain (equivalent to total protein from four larvae); lanes 2–9, intracellular proteins; lanes 11–13, media-excreted proteins; lanes 2–3, LacZ-positive control 0 and 24 h after induction of expression with methanol; lanes 4–6, pPICZ α A no insert negative control 0, 24, and 48 h after methanol induction; lanes 7–9, *CzEst9*-pPICZ α A construct expression 0, 24, and 48 h after methanol induction in MMH; lanes 11–13: *CzEst9*-pPICZ α A construct expression 0, 24, and 48 h after induction in BMMY. (B) Native esterase activity gel of nickel-affinity column captured (lanes 2, 4, and 6) and flow-through (lanes 3, 5, and 7) fractions. Lane 1, total soluble native proteins from Cz strain (equivalent to total protein from 0.8 larvae); lanes 2–3, intracellular proteins from GS115/pPICZ//lacZ Mut⁺ β -galactosidase-expressing induction control; lanes 4–5, media-excreted proteins from pPICZ α A no insert negative control; lanes 6–7, *CzEst9*-pPICZ α A recombinant construct. (C) Western blot of SDS-PAGE gel of nickel-affinity column captured (lanes 1, 3, and 5) and flow-through (lanes 2, 4, and 6) fractions probed with anti-His-HRP antibody. Locations of the 250-, 150-, 100-, 75-, 50-, and 37-kDa molecular weight markers are noted on the left of the image. Lanes 1–2, intracellular proteins from GS115/pPICZ//lacZ Mut⁺ β -galactosidase-expressing induction control; lanes 3–4, media-excreted proteins from pPICZ α A no insert negative control; lanes 5–6, media-excreted proteins from *CzEst9*-pPICZ α A His-tagged recombinant construct.

recombinant protein into the growth media (data not shown). Although the *E. coli* systems produced a satisfactory amount of CzEST9, the protein was consistently found in the inclusion body fraction of the cells. This necessitated protein purification under conditions which resulted in CzEST9 inactivation. Several renaturation protocols were attempted that all failed

to reconstitute esterase activity (data not shown). However, the *CzEst9*-pPICZ α A vector transformed into *P. pastoris* GS115 produced a methanol-inducible intracellular recombinant protein that possessed esterase activity on native esterase activity gels and migrated on these gels at a similar rate as CzEST9 from resistant tick larvae (Fig. 3A). This intracellular pro-

tein was present at 24 and 48 h after recombinant expression induction with methanol in MMH media (Fig. 3A, lanes 8–9, arrow) and BMMY media (data not shown). This esterase activity was not seen in either a methanol-inducible GS115/pPICZ/*lacZ* Mut⁺ intracellular β -galactosidase-expressing control or a pPICZ α A (no insert) negative control (Fig. 3A, lanes 2–6). All constructs produced an additional band of intracellular esterase activity 24 and 48 h after methanol induction; however, this faster migrating product was easily resolvable from the putative recombinant CzEST9 activity (Fig. 3A, lanes 2–9). When the MMH and BMMY media-excreted protein fractions were examined on native esterase activity gels, a methanol-inducible band corresponding to that expected for the CzEST9 recombinant protein was found at the 48 h postinduction time when BMMY media were used (Fig. 3A, lanes 11–13). When the cells were grown and induced in MMH media, no proteins with esterase activity were noted in the media-excreted fractions (data not shown). Additionally, the faster migrating band with esterase activity seen in the intracellular fractions (Fig. 3A, lanes 2–9) was not seen in any of the media-excreted protein samples. This unknown esterase activity was always seen in the intracellular samples after methanol induction, sporadically and usually at much reduced levels in samples taken before methanol induction, and never in the media-excreted protein samples (data not shown). To verify the identity of the putative CzEST9 recombinant, intracellular proteins from the CzEST9-pPICZ α A recombinant expression construct, the GS115/pPICZ/*lacZ* Mut⁺ β -galactosidase-expressing control, and the pPICZ α A no insert negative control were fractionated on a nickel-affinity chromatography minicolumn after methanol induction of the growth cultures, and both the flow-through and the eluted affinity-captured fraction analyzed by native esterase activity gel electrophoresis (Fig. 3B). Although the protein yield from the minicolumns seemed low, the only sample with esterase activity on these activity gels was that containing the CzEST9-pPICZ α A recombinant expression construct (Fig. 3B, lanes 6–7, arrow). The nickel-captured fraction contained a single band of esterase activity that migrated similarly to CzEST9 from tick larvae (Fig. 3B, lane 6, arrow). The flow-through fraction (Fig. 3B, lane 7) contained two bands of esterase activity that did not migrate at the same rate as the CzEST9 from tick larvae. SDS-polyacrylamide gel electrophoresis (PAGE) of both the nickel-captured and flow-through fractions corresponding to the samples analyzed in Fig. 3B, followed by Western blotting with anti-His-HRP antibody verified that His-tagged protein was present only in the nickel-captured fractions of both the β -galactosidase-expressing His-tagged control and the CzEST9-pPICZ α A His-tagged recombinant (Fig. 3C, lanes 1 and 5). Both the β -galactosidase-expressing control and the CzEST9-pPICZ α A recombinant proteins migrated at rates similar to their respective calculated molecular weights of 119 and 74.2 kDa. Thus, adopting the *P. pastoris* system led to successful expression of recombinant protein

with esterase activity. Several different growth conditions were attempted to increase the yield of recombinant protein, including growth and induction at 15°C in the presence of supplemental yeast extract and glycerol, which Woo et al. (2004) reported to enhance the expression of anti-T-cell immunotoxin A in *P. pastoris*. Our attempts did not appreciably increase the recombinant protein yield. Production of larger quantities of CzEST9 from our clone would require scaling up culture size, perhaps using fermentation processes for which *P. pastoris* is well suited. Another alternative would involve screening for transformants possessing multiple copies of CzEST9, a strategy reported by Clare et al. (1991) to lead to higher recombinant protein expression levels in *P. pastoris*.

Despite not finding CzEST9 resistance-associated promoter elements in this study, the determination of the CzEST9 gene structure makes available an arachnid gene promoter region from *R. microplus* ticks that would be useful in genetic transformation experiments with ticks or tick cell lines. Promoter analysis based on searching for binding motifs in the primary sequence has limited value because of the high proportions of predicted binding sites that bind *in vitro* but are not functional *in vivo* (Wasserman and Sandelin 2004). However, as sequence information becomes available from the *Ixodes scapularis* Say genome project, it will be interesting to see if CzEST9-like genes are present in that tick and to make comparisons between the gene promoters of the esterase genes from *I. scapularis* and CzEST9. The promoter sequence information, coupled with reagents such as a metabolic esterase gene microarray chip, which could be developed from the genome sequence or expressed sequence tag projects from various tick species, might enable the identification of *cis*-acting DNA sequence promoter elements functioning in response to metabolic perturbances, such as exposure to pesticides. This information might lead to insight useful for the development of novel acaricides or methods to extend the usefulness of current acaricides which might have problems due to the presence of metabolic esterase detoxification mechanisms, such as exists in the Cz strain of *R. microplus*.

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